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Reduced salinities negatively impact fertilization success and early larval development of the giant clam *Tridacna gigas* (Cardiidae: Tridacninae)

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ABSTRACT

The increasing frequency and intensity of precipitation attributed to global climate change, in combination with sewage disposal, agricultural runoff, and other anthropogenic sources, could lead to a reduction in the salinity levels of shallow water reefs. Given that many reef animals including the giant clam exhibit a bipartite life cycle wherein fertilization and larval development take place in the water column prior to settlement, tridacnine early life stages are susceptible to reduced salinity levels. To investigate the impacts of reduced salinities on the early life stages of the giant clam, we exposed the gametes, embryos, and larvae of *Tridacna gigas* to salinities of 18, 22, 26, 30, and 34 ppt. Fertilization occurred in all treatments but was significantly reduced at 18 ppt. Lower salinity conditions resulted in delayed embryonic development, with fewer trochophore and veliger larvae at 18, 22, and 26 ppt. No larvae survived more than two days of exposure at 18 ppt and four days at 22 or 26 ppt. Salinity did not have a significant effect on pediveliger larvae settlement rate or shell length. This study shows that reduced salinity affects both the fertilization success and survival of developing tridacnine larvae, suggesting that intensified precipitation events could further inhibit the survival of giant clams on the reef.

1. Introduction

Giant clams (Bivalvia: Cardiidae: Tridacninae) are important components of coral reef ecosystems as reef builders and providers of food and habitat to many marine organisms (Cabaitan et al., 2008; Neo et al., 2015a). However, giant clam populations have been depleted because of environmental and anthropogenic disturbances such as over harvesting and habitat destruction (Gomez and Mingoa-Licuanan, 2006; Neo et al., 2017). These disturbances resulted in a decline of giant clam stocks to densities insufficient to self-replenish and maintain their populations, which might eventually lead to extinction if not given attention (Munro, 1992; Neo and Todd, 2013). Giant clams are classified as endangered by the International Union for the Conservation of Nature (IUCN) (Neo and Todd, 2013) and are protected under the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2016).

Among the common natural and anthropogenic disturbances, reduced salinity is one of the major physiological stressors of marine animals including giant clams (Brown, 1997; Hoegh-Guldberg and Smith, 1989; Blidberg, 2004). Stenohaline marine animals have limited tolerance to changes in salinity and may experience drastic alterations in their physiological state due to osmotic stress (Anger, 2003). Numerous studies have demonstrated the impacts of reduced salinities across various marine taxa groups. Specifically, a reduced salinity of 25.8 ppt decreased fertilization success and subsequent development of coral larvae (Chui et al., 2016; Scott et al., 2013), while adult corals exhibited bleaching and mortality at a salinity of 20 ppt and lower (Chavanich et al., 2009; Faxneld et al., 2010; Jokiel et al., 1993). In other invertebrates, such as the sea urchin *Parechinus angulosus*, decreased fertilization success at salinity < 15 ppt occurred due to damage to the ova (Greenwood and Bennett, 1981). Larval development, growth, survival, and filtration rate of bivalve species such as the oyster *Crassostrea iredalei* are also adversely affected by salinity lower than 15 ppt (Chang et al., 2016; Fang et al., 2016), while 10 ppt or lower is often considered lethal for larvae of the bay scallop *Argopecten irradians irradians* (Tettelbach and Rhodes, 1981).

Giant clams exhibit differences in spawning seasonality across regions, but spawning is reported to peak during summer months (Beckvar, 1981; Braley, 1984; Soo and Todd, 2014). Spawning is commonly observed mid to late afternoon on a rising tide near new and full moon phases (Jameson, 1976; Beckvar, 1981; Braley, 1984). Giant clams are broadcast spawners and exhibit a bipartite life cycle where their gametes and pelagic larvae spend approximately nine days in the water column prior to settlement (Lucas, 1988). This behavior makes

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the early life stages susceptible to environmental changes such as reduction in seawater salinity (Blidberg, 2004; Marshall et al., 2016). Survival and tolerance of the early life stages of *T. squamosa* (Eckman et al., 2014; Neo et al., 2013) and juveniles of *T. gigas* (Maboloc et al., 2014, 2015; Rachman and Anshary, 1997) to a hyposaline environment have been previously examined. Survival and development of *T. squamosa* trochophore larvae were not affected at 27 and 30 ppt for up to 24 h (Neo et al., 2013), trochophores survived up to 3 h at 9 ppt, and veligers survived up to 42 h at 12 ppt (Eckman et al., 2014). Similarly, the growth of juvenile *T. gigas* was affected at 27 ppt (Rachman and Anshary, 1997), while total mortality was observed at 18 ppt (Maboloc et al., 2015). Apart from a single study demonstrating lower survival of *T. gigas* veliger larvae at 20 and 25 ppt (Blidberg, 2004), no other studies have been conducted on the effect of salinity on the early life stages of *T. gigas*.

Nowadays, reduction in salinity is becoming common due to the increasing number of flood events, river run offs, and submarine ground water discharge associated with intense precipitation due to global climate change, as well as the freshwater inputs from unregulated anthropogenic activities (Brown, 1997; Morton, 2002; Kerswell and Jones, 2003; Burdett et al., 2015; Chui et al., 2016). Frequent heavy rainfall events cause lower salinity in shallow waters, especially near river mouths and in coastal areas (Cardenas et al., 2010; Maboloc et al., 2014). The predicted rise in frequency of extended rainfall events, tropical storms and typhoons in the future underlines the importance of understanding the impacts of reduced salinity on the development and physiology of coral reef organisms, including giant clams.

In the Philippines, hatchery-bred *Tridacna gigas* is being restocked around the country to bring back their populations in the wild (Gomez and Mingoa-Licuanan, 2006) but so far, only a few recruits have been observed near restocking sites (Cabaitan and Conaco, 2017). Although various factors could contribute to the low local recruitment and survival rates, reduced salinity is likely a driving factor because most restocking sites are located in shallow waters close to nearshore areas that are affected by frequent and intense rainfall (e.g., typhoon) even in summer months (Cinco et al., 2014; Cinco et al., 2016; Villafuerte et al., 2014) when clams are expected to spawn. Thus, there is an urgent need to assess the physiological responses of *T. gigas* gametes and larval stages to a range of reduced salinities. This knowledge will help in understanding their tolerance thresholds, as well as possible coping mechanisms, with implications on giant clam resilience and adaptation to a changing environment. The objectives of the study are to determine the effects of reduced salinities on various aspects of the early life stages of *T. gigas*, specifically examining the fertilization success, development, survival and tolerance of pelagic larvae, and settlement.

2. Materials and methods

2.1. Induced spawning and larval rearing

Spawning and larval rearing were adapted from the protocols described by several authors (Braley, 1992; Crawford et al., 1986; Mingoa-Licuanan and Gomez, 2007). Mature *T. gigas* broodstock from two different cohorts were collected from the Silaqui Giant Clam Ocean Nursery (16°26.806′N, 119°55.352′E), located at a back reef offshore of Bolinao, Pangasinan, in northwestern Philippines. Giant clams selected as sperm donors were 9 years old with a shell length of 49.57 ± 0.58 cm [mean ± SE] while giant clams selected as egg donors were 17 years old with a shell length of 61.27 ± 0.86 cm. As individuals may release gametes of varying quantity and quality, five clams of each cohort were collected to ensure that sufficient numbers of gametes would be obtained for the experiments. The clams were brought to the outdoor hatchery of the Bolinao Marine Laboratory (BML) of the Marine Science Institute, University of the Philippines, for ex situ spawning. Spawning was conducted at midday during the new moon phase. Clams were induced to spawn by injecting 4.0 mL of 2 mM serotonin solution (serotonin creatinine sulfate monohydrate, Sigma H7752-1G) around 2 cm below the excurrent siphon into their gonads (Braley, 1985; Mingoa-Licuanan and Gomez, 2007). Eggs and sperm from each individual were collected upon release and immediately viewed using a microscope to check for gamete quality. Only spherical eggs and milky and homogenous sperm were used in the experiment (Mingoa-Licuanan and Gomez, 2007). For larval rearing, eggs pooled from three giant clam egg donors were fertilized by the sperm pooled from three giant clam sperm donors, following the egg-sperm ratio of 1 L:5 mL. Fertilized eggs were stocked in fiberglass conical tanks (300 L) following the recommended density of 10–20 individuals mL^{-1} (Mingoa-Licuanan and Gomez, 2007). The entire rearing water was changed every two days to ensure optimum culture conditions. Streptomycin sulfate (10 mg L^{-1}) was added right after fertilization and after the rearing water was changed to prevent bacterial proliferation (Braley, 1992). *Tetraselmis* sp. algae (10,000 cells mL^{-1}) was provided daily starting on the second day when larvae start to filter feed, while zooxanthellae (100 cells mL^{-1}) isolated from juvenile *T. gigas* was introduced every two days starting on the third day of larval culture (Mingoa-Licuanan and Gomez, 2007). Zooxanthellae were isolated by homogenizing the mantle of juvenile *T. gigas* then filtering the homogenate through a 60 μm mesh sieve to remove tissue debris.

2.2. Salinity treatments and rearing conditions

Five levels of salinities were tested: 18, 22, 26, 30, and 34 ppt. The latter represents the ambient spawning salinity. Salinities from 30 to 26 ppt have been observed at the Bolinao Marine Laboratory hatchery facility, as well as at the Silaqui giant clam ocean nursery, in Bolinao, Pangasinan, northwestern Philippines, following heavy rainfall events (Maboloc et al., 2014). Salinities of 22 to 18 ppt were reported at the Great Barrier Reef (GBR) in Australia following heavy rainfall and high flood events (Buck et al., 2002; Berkelmans et al., 2012; Jones and Berkelmans, 2014). The different salinity levels were attained by mixing 5 μm-filtered and UV-treated seawater (UVFSW) with freshwater and checked using a handheld refractometer (Atago).

Three experiments were conducted; (a) *Fertilization success, larval development and survival over time*, (b) *Acute exposure and tolerance of post-fertilization embryos and larvae*, and (c) *Settlement of larvae*. Experiments were conducted in 1.1 L plastic containers filled with 1 L water and partially immersed in a water bath to minimize temperature fluctuations in the small containers. The water bath was kept at ambient temperature with flow-through seawater pumped directly from the ocean. Mild aeration was provided using small air stones to keep constant water circulation in each container. Water parameters such as dissolved oxygen, pH, and salinity were measured from each container every day at 8:00 am and 4:00 pm using DO meter (Milwaukee), pH meter (PH-200, HM Digital), and handheld refractometer (Atago), respectively. Salinity values were immediately adjusted when changes were detected after addition of algae during feeding. Temperature and light intensity were measured at five-minute intervals using a submersible HOBO logger. A different set of experimental containers were prepared for each type of observation under each experiment, unless otherwise described below, and each sampling time point was represented by three separate replicate containers.

2.3. Fertilization success, larval development and survival over time

Around 10,000 eggs were filtered onto a 60 μm plankton net and washed twice with UVFSW. The eggs were washed off the filter and into the experimental container using 1 L of seawater at the corresponding salinity and immediately fertilized with 5 mL sperm solution. Rearing procedures closely followed those described in Braley (1992), Crawford et al. (1986) and Mingoa-Licuanan and Gomez (2007) as mentioned in Section 2.2. Three replicate containers were prepared for each sampling point. Each replicate was only sampled once and then discarded. To

determine fertilization success, eggs were filtered from each of 3 replicate containers per salinity treatment at 3 and 6 h post fertilization (hpf). The number of fertilized eggs out of the first 30 individuals encountered per sample was counted. The fertilization experiment lasted for six hours, by which time all eggs were expected to have undergone multiple cell divisions before gastrulation. Eggs that had undergone either regular or irregular cleavage at the time of observation were considered fertilized, while eggs that remained spherical in shape were categorized as unfertilized (Enricuso et al., 2018).

To quantify survival, all live embryos or larvae were counted from three 1 mL subsamples taken from each of 3 replicate containers per salinity treatment at 3, 6, 12, 24, 48, 96, 144, and 192 hpf. The number of live embryos or larvae in each replicate container was taken as the mean count from the 3 subsamples. After sampling for the quantitation of larval survival, the remaining embryos or larvae in each 1 L container were collected for the analysis of development. Larvae were filtered through a 60 μm plankton net and transferred into separate 10 mL vials containing seawater at the corresponding salinity level. Five drops of 10% formalin in seawater was added to stop further development of the larvae and to preserve the sample. A sample was taken from each vial and the proportion of eggs, embryos, and larvae at various developmental stages was determined from the first 30 individuals counted per sample. The experiment for larval development and survival lasted for eight days until larvae reached the pediveliger stage and were ready to settle (Jameson, 1976; Soo and Todd, 2014; Southgate et al., 2016). All individuals in good condition, which include the spherical egg, cleaved embryo (e.g., regular and irregular), ciliated gastrula, trochophore, veliger and pediveliger larvae were considered as alive, while the degraded or lysed bodies were categorized as dead. All samples were viewed and examined using a Sedgewick-Rafter slide and compound microscope (Motic Zeiss).

2.4. Acute exposure and tolerance of post-fertilization embryos and larvae

Gametes were fertilized and cultured at ambient condition (34 ppt) at 10 individuals mL^{-1} in a 300 L conical fiber glass tank following the recommended rearing conditions (Braley, 1992; Crawford et al., 1986; Mingoa-Licuanan and Gomez, 2007) until the experiment commenced. Individuals at 3 hpf and at 24 hpf were collected from the rearing tank for the experiment. They were filtered onto a 60 μm plankton net and washed off from the filter using seawater of the corresponding salinity treatment until the experimental container was filled to 1 L. The recommended stocking density of 10 individuals mL^{-1} and larval rearing protocols were followed (Braley, 1992; Crawford et al., 1986; Mingoa-Licuanan and Gomez, 2007). Sampling for development of 3 hpf individuals was done at 12 (i.e., 9 h later), 24, 48, 96,144 and 192 hpf, while for 24 hpf individuals, sampling was at 48 (i.e., 24 h later), 96, 144 and 192 hpf. Categorization of larval development and sampling for survival and developmental success was similar to the procedures mentioned above (Section 2.3). The experiment was terminated once larvae reached the pediveliger stage.

2.5. Settlement of larvae

Larvae were considered to have settled and metamorphosed once they had lost their ability to swim, which usually occurs between 6 and 14 days of culture (Ellis, 1998; Neo et al., 2015b; Southgate et al., 2016). Eight-day old pediveliger larvae reared under ambient conditions were used in this experiment. Concrete settlement substrata of the same material as the settlement raceways used for giant clam culture at the Bolinao Marine Laboratory were provided to the settling pediveligers. Prior to the experiment, settlement substrata (12 cm diameter) were cleaned with freshwater and soaked in flowing filtered seawater for three days to condition the substrate surface and eliminate residual chemicals from the cement (Neo et al., 2009). The substratum was then fitted onto the bottom of the 1.1 L circular plastic containers. Containers were half immersed in a water bath supplied with flowing seawater at ambient temperature. Pediveliger larvae were added into each container at a density of ~5 individuals cm^{-2}. On the second day of experiment, 50% of rearing water was changed before feeding to minimize disturbance to the settling larvae. Larvae were fed daily with 15,000 cells mL^{-1} of *Tetraselmis* sp. while zooxanthellae were provided at 100 cells mL^{-1} on the first and third days of culture. The presence of swimming larvae was checked on the third and fourth day of culture by taking five 1 mL subsamples from different areas in each container. Survival of settled clams was quantified only after four days when swimming larvae were no longer detected in the water column. To facilitate counting, settled juveniles were washed off the substratum, filtered using 100 μm net and concentrated into a 20 mL vial. The number of live and dead juveniles was determined as the mean count of three 1 mL subsamples per vial. Empty and transparent shells were considered dead while individuals with evident internal organs seen through its transparent shell were considered alive. In addition, the shell length of ten live individuals pooled from three replicates per salinity treatment was measured.

2.6. Statistical analyses

All data obtained fulfilled the assumptions of normality and parametric analyses were used. Variability in the percent fertilization success and the survival of *T. gigas* exposed to the five salinity treatments across time were separately analyzed for each experiment using two-way ANOVA. One-way ANOVA was used to analyze mean percent settlement and shell size among the five salinity treatments. *Post-hoc* Tukey's HSD tests were conducted on ANOVA results that were significant. Alpha level of significance of 0.05 was used. All statistical analyses were carried out using Statistica 8.

3. Results

Salinity levels in rearing containers were maintained all throughout the experiment (Table 1). Other physico-chemical parameters (i.e., DO, pH, temperature, and light intensity) measured during the experiment are presented in Table 1.

Table 1
Physico-chemical parameters in the experimental containers. Average values ± SE over the course of the experiments are shown.

Parameters	Salinity treatments (ppt)				
	34 (control)	30	26	22	18
Salinity (ppt)	33.94 ± 0.04	29.83 ± 0.02	26.06 ± 0.04	22.0 ± 0.00	18.07 ± 0.09
pH	8.33 ± 0.00	8.34 ± 0.01	8.33 ± 0.00	8.25 ± 0.01	8.28 ± 0.04
Dissolved oxygen ($mg\,L^{-1}$)	6.11 ± 0.06	5.81 ± 0.14	5.66 ± 0.12	5.72 ± 0.10	5.37 ± 0.10
Temperature (°C)	26.31 ± 0.04	26.43 ± 0.04	26.48 ± 0.04	26.50 ± 0.04	26.41 ± 0.04
Light intensity (lux)	349.7 ± 9.42	344.70 ± 9.26	367.42 ± 9.93	345.64 ± 9.31	308.51 ± 8.32

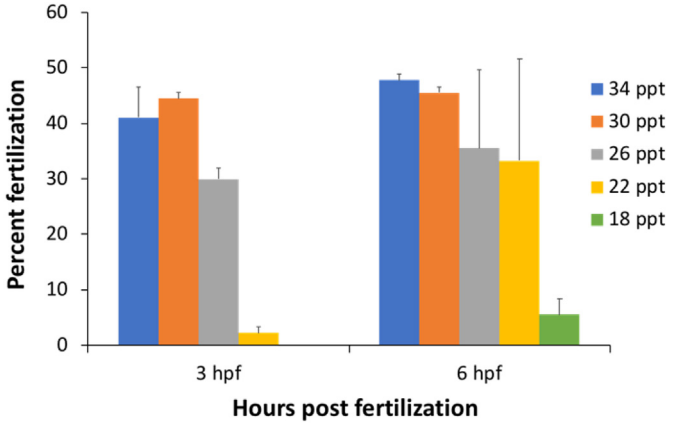

Fig. 1. Effect of salinity on the fertilization success of *T. gigas* after 3 h and 6 h of exposure (mean ± SE, $n = 3$).

3.1. Fertilization success

Fertilization was observed at all salinities tested as indicated by the presence of cleaved embryos (Fig. 1). However, at 18 ppt, cleavage was observed only 6 h after gametes were mixed. At 3 hpf, only 2.22 ± 1.11% (mean ± SE, $n = 3$) of individuals at 22 ppt exhibited cleavage compared to 30–44% fertilization in the higher salinity treatments. At 6 hpf, 5.56 ± 2.94% of eggs at 18 ppt and 33.33 ± 18.36% at 22 ppt showed signs of successful fertilization. Fertilization success at 18 and 22 ppt was significantly lower compared to 26 ppt (35.56 ± 14.19%), 30 ppt (45.56 ± 1.11%) and 34 ppt (47.78 ± 1.11%) (Tukey's HSD test: $p < 0.05$).

3.2. Larval development and survival over time

At 12 hpf, many individuals had completed gastrulation but the proportions of gastrula at 18 ppt (28.89 ± 2.94%) and 22 ppt (32.22 ± 1.11%) were lower compared to those at 26 ppt (57.78 ± 11.76%), 30 ppt (62.22 ± 4.0%), and 34 ppt (65.56 ± 9.09%) (Tukey's HSD test: $p < 0.05$; Fig. 2). Trochophore larvae were first observed after 24 hpf at salinities of 26, 30 and 34 ppt while at 22 ppt, this stage appeared only after 48 hpf. No trochophores were observed at 18 ppt, suggesting 100% mortality. Veliger larvae were first observed after 48 hpf at both 30 and 34 ppt. At 96 hpf, all individuals at 34 ppt had developed into veligers, whereas at 30 ppt, trochophore and ciliated gastrula were still present. Larvae at 30 and 34 ppt metamorphosed to the pediveliger stage by 192 hpf.

Survival of larvae significantly decreased with decreasing salinity treatments throughout the 8-day experiment (192 h) (Two-way ANOVA: Treatment*Time, $F_{32,\ 90} = 6.22$, $p < 0.05$; Fig. 3). Embryos maintained at 18 ppt did not survive past 48 hpf while those at 22 ppt and 26 ppt did not survive past 96 hpf. No significant difference was found between 30 ppt and 34 ppt starting at 48 hpf until the end of the experiment at 192 hpf (Tukey's HSD tests: $p > 0.05$).

3.3. Salinity tolerance of post-fertilized embryos and larvae

Three hour-old individuals exhibited 100% mortality when subjected to a salinity of 18 or 22 ppt for 93 h and at 26 ppt for 141 h (Fig. 4a). Embryos in the low salinity treatments developed up to trochophore stage only, with the exception of a small proportion of larvae that metamorphosed into veligers at 26 ppt (17.78 ± 10.6%). In contrast, 24 h-old individuals survived for up to 120 h in all salinity treatments, incurring 100% mortality only after 168 h at salinities of 26 ppt and below (Fig. 4b). The 24 h-old individuals maintained at reduced salinities developed to veliger stage. All individuals subjected at 30 and 34 ppt survived and developed up to the pediveliger stage.

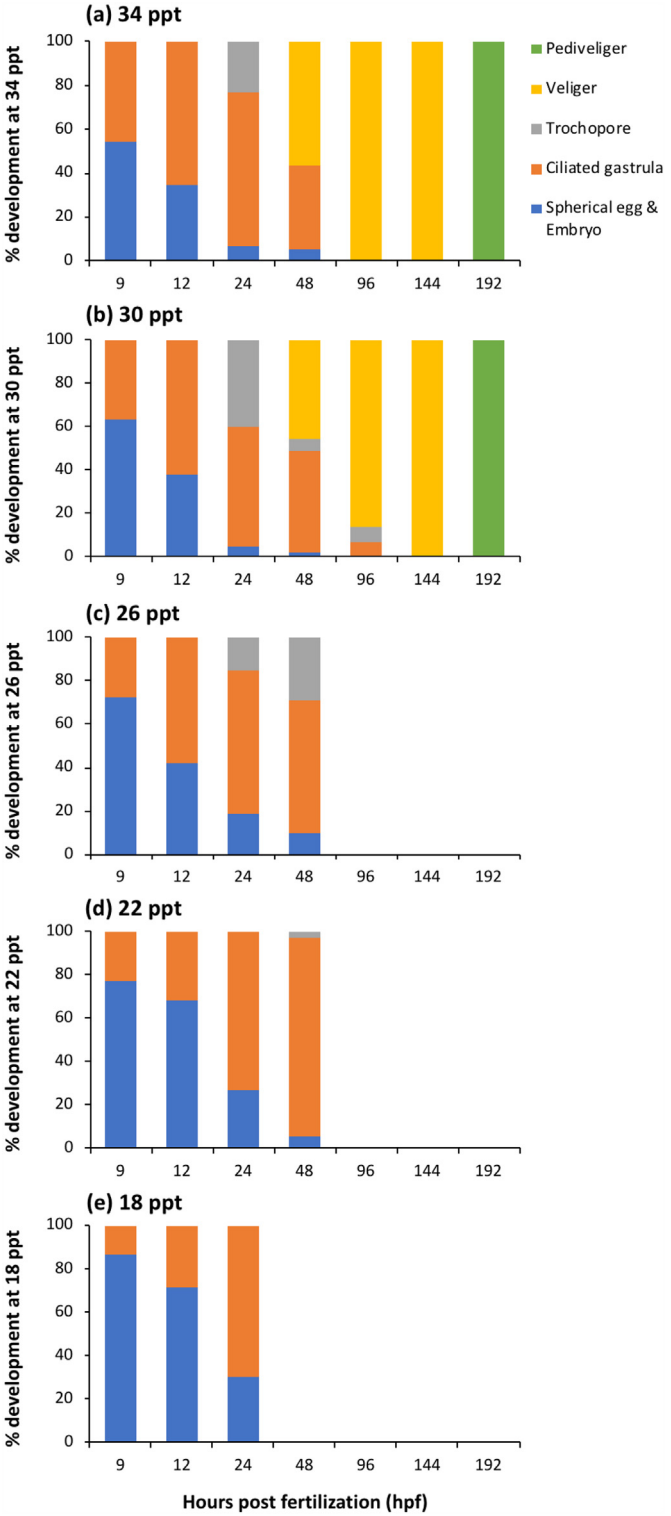

Fig. 2. Development of *T. gigas* at different salinities. The proportion of early developmental stages observed from 9 to 192 hpf at salinities of (a) 34 ppt, (b) 30 ppt, (c) 26 ppt, (d) 22 ppt and (e) 18 ppt is shown. Each 100% stacked bar represents the mean proportions from three replicates at each time point.

3.4. Settlement of larvae

Pediveliger larvae settlement was generally higher at higher salinity levels (Fig. 5): 34 ppt (50.0 ± 6.5%), 30 ppt (40.4 ± 7.2%), 26 ppt (38.5 ± 4.8%), 22 ppt (38.9 ± 4.4%), and 18 ppt (24.1 ± 6.4%). However, the proportion of settled larvae was not significantly different

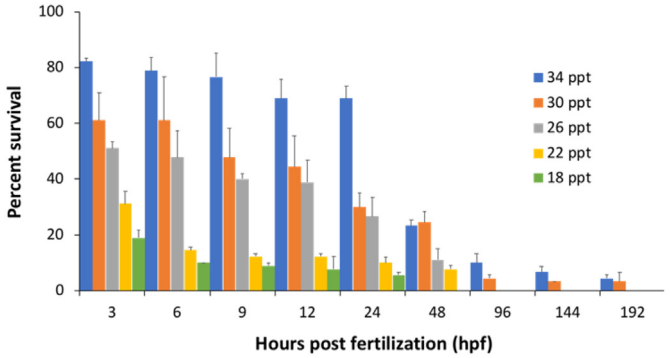

Fig. 3. Percent survival of *T. gigas* larvae maintained at different salinities from 0 to 192 hpf (mean ± SE, $n = 3$). Survival varied across salinities through time (Two-way ANOVA: Treatment*Time interaction, Tukey's HSD test, $F_{32, 90} = 6.22$, $p < 0.05$).

across salinity treatments (One-way ANOVA: $F_{4, 10} = 2.41$, $p > 0.05$). Moreover, no significant differences in shell size were observed in juveniles reared at different salinities (Fig. 6): 34 ppt (174.4 ± 2.0 μm), 30 ppt (175.3 ± 2.6 μm), 26 ppt (178.8 ± 3.4 μm), 22 ppt (182.8 ± 2.5 μm), and 18 ppt (182.8 ± 2.1 μm) (One-way ANOVA: $F_{4, 45} = 2.19$, $p > 0.05$).

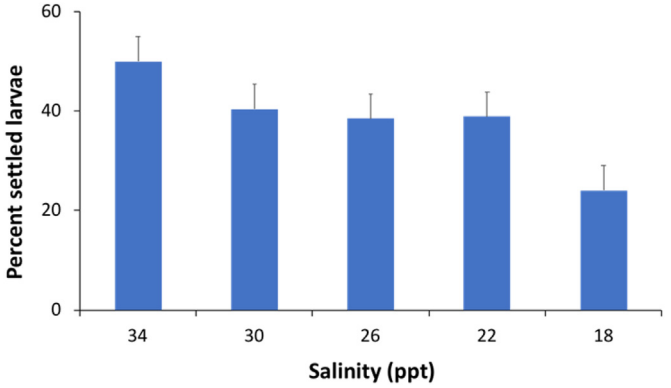

Fig. 5. Percent settlement of *T. gigas* pediveliger larvae after 4 days of exposure to different salinities (mean ± SE, n = 3). Percent settlement was not significantly different among treatments (One-way ANOVA, $F_{4, 10} = 2.41$, $p > 0.05$).

4. Discussion

4.1. Fertilization success

Fertilization was observed at all salinity levels tested. However, fertilization success was reduced and development was delayed at lower salinities. Formation of the fertilization envelope upon contact between sperm and eggs may have provided some protection against environmental stressors, pathogens, and toxins (Wong and Wessel, 2008), thus helping the embryo maintain normal cellular functions. The

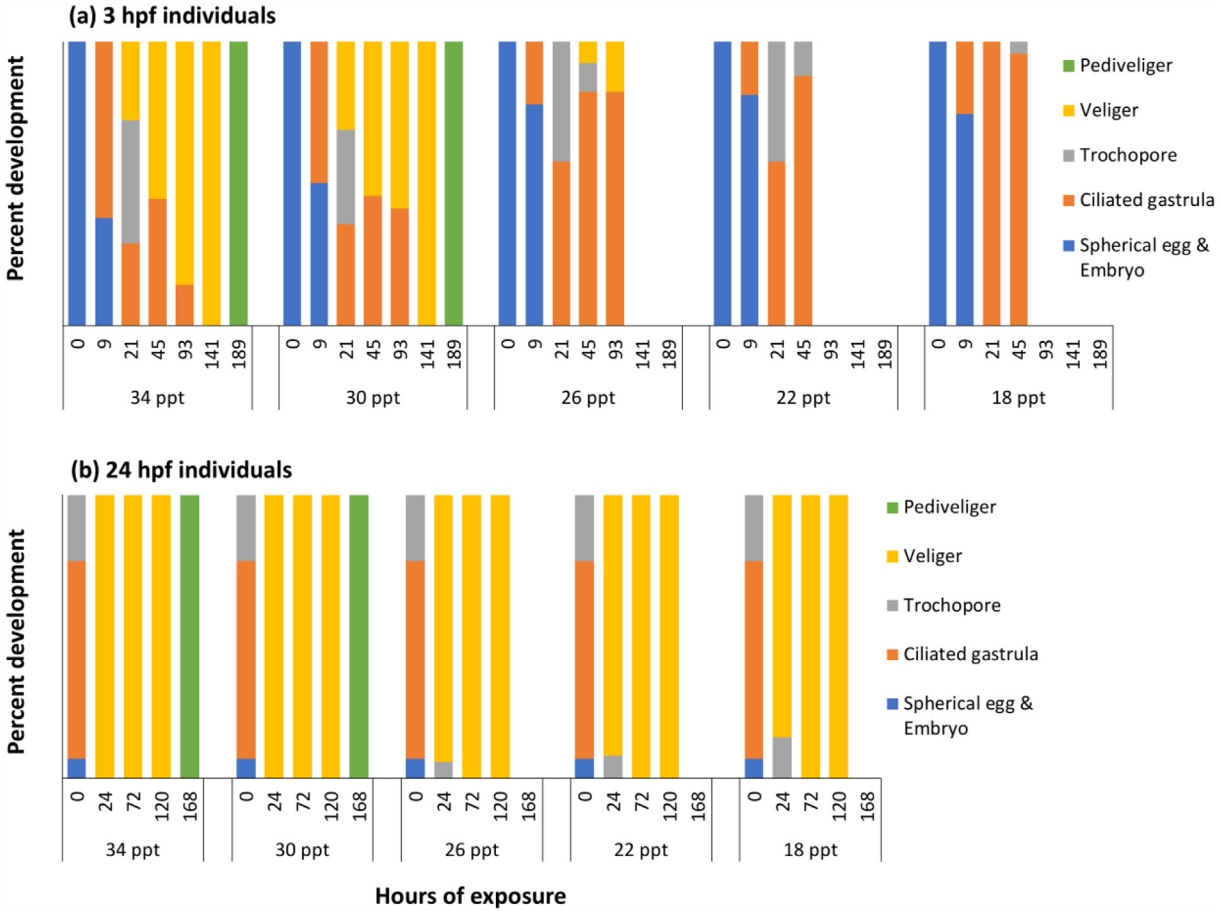

Fig. 4. Tolerance of early developmental stages to low salinity exposure. Percent survival and development of larvae derived from (a) embryos (3 hpf) or (b) larvae (24 hpf) subjected to reduced salinities for different durations.

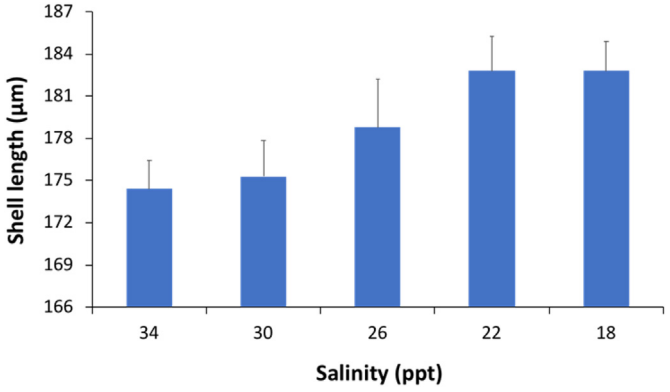

Fig. 6. Shell length of *T. gigas* pediveligers after 4 days of exposure at different salinities (mean ± SE, $n = 10$). Shell length was not significantly different among treatments (One-way ANOVA: $F_{4, 45} = 2.19$, $p > 0.05$).

marked delay in embryonic cleavage could be associated with reduced activation of the mitosis promoting factor (MPF) in a less saline environment (Ciapa and Philippe, 2013). The delay could also be attributed to reduced sperm motility under hyposaline conditions (Caballes et al., 2017). Furthermore, deterioration of egg quality due to low salinity exposure may have contributed to reduced fertilization rate, as has been demonstrated in other marine invertebrates (Greenwood and Bennett, 1981; Qiu et al., 2002; Kashenko, 2000, 2007a,; Pechenick et al., 2007; Allen and Pechenick, 2010; Delorme and Sewell, 2014). The inability to control the inflow and outflow of water in the cells (Greenwood and Bennett, 1981) and the swelling of the hyaline layer due to osmotic stress (Allen et al., 2015) may explain the degradation of gametes.

4.2. Embryonic, and larval development and survival through time

Environment stressors, such as reduced salinity, can delay the process of development and reduce overall survival rate (Blidberg, 2004; Rahman et al., 2014). At salinities of 18 and 22 ppt, embryos exhibited a delay in hatching from the intact fertilization envelope, which may be due to a failure to produce the hatching enzyme, a protease needed for further development (Lepage and Gache, 1989, 1990). This delay could be a means of preventing undue exposure of the developing embryo to reduced salinity, as has been reported in the sand dollar *Echinarachnius parma* (Armstrong et al., 2012). In some cases, however, development of embryos even to blastula stage does not proceed, as observed in the Pacific mussel *Crenomytilus grayanus* when exposed to 18 to 26 ppt (Yaroslavtseva and Sergeeva, 2009). Late production of cilia and loss of ciliary beating and locomotion could further contribute to delays or cessation of embryonic development (Wells and Ledingham, 1940; Kashenko, 2007b; Armstrong et al., 2012). Also, the inability to develop the first hinge inhibits further shell development (LaBarbera, 1974). In *T. gigas*, a greater number of pelagic larvae remained at 20 and 25 ppt when most larvae at 32 and 34 ppt had already undergone metamorphosis, further indicating that salinity stress can result in developmental delay (Blidberg, 2004). Similar observations have been reported in other marine organisms subjected to salinity stress, including the bivalve *Crassostrea iredalei* (Fang et al., 2016), sea urchins *Echinocardium cordatum* (Kashenko, 2007a) and *Heliocidaris crassispina* (Mak and Chan, 2018), sand dollars *Scaphechinus mirabilis* (Kashenko, 2009) and *E. parma* (Armstrong et al., 2012), and crown-of-thorns seastar *Acanthaster* cf. *solaris* (Allen et al., 2017). Our findings are contrary to the results of studies in *T. squamosa* showing that development of embryos and trochophore larvae are not affected by exposure to salinities of 27–30 ppt for 3 to 24 h (Neo et al., 2013) or to 0–12 ppt for 10 min to 42 h (Eckman et al., 2014). However, the embryos used by Neo et al. (2013) and Eckman et al. (2014) were cultured under ambient conditions prior to experiments and were only exposed in reduced salinities for shorter durations, compared to current study. Furthermore, *T. squamosa* used in those studies were from Singapore waters wherein marine organisms are likely to experience hyposaline conditions (Eckman et al., 2014), which may explain why *T. squamosa* can better tolerate hyposaline conditions.

Tridacna gigas embryos and larvae maintained at lower salinities (18, 22, and 26 ppt) showed abnormal development (i.e., irregular in shape, deformed larvae), degradation, and eventual mortality. Abnormalities in larval development at low salinity could be due to changes in cellular osmotic balance, which can cause changes in form, similar to the report of Kashenko (2009) where sand dollar larvae had a more spherical form at 18 and 20 ppt. Swelling of the hyaline layer, the extracellular matrix surrounding the developing embryo (Allen et al., 2015), and the increase in metabolic activity of larvae for osmoregulation (Anger, 2003) can lead to mortality. In addition, the lower sodium concentration of seawater induces intracellular acidification that inhibits the cell cycle and lead to cell death (Ciapa and Philippe, 2013). Furthermore, in times of stress, the increased generation of reactive oxygen species (ROS) can cause oxidative damage to embryos and larvae (An and Choi, 2010). This present study is similar to the report of Blidberg (2004) wherein survival of veliger larvae significantly decreased at 20 and 25 ppt.

Although survival of larvae at the 34 ppt (ambient) treatment was low (4.44%), this value was comparable to the 5% survival recorded in the standard larval rearing tanks for the same set of larvae that were used in this present study. Additionally, regardless of salinities tested, it was evident that the greatest decline in survival was observed between 24 hpf and 48 hpf, which coincides with larval metamorphosis from trochophore to veliger (Fig. 3). This observation is similar to the report of Yamaguchi (1977) and Braley et al. (1988), where high mortality is typically observed during the early pelagic and pre-veliger stages. A possible explanation could be due to the larvae being more susceptible to stressors at this stage as they transition to planktotrophic feeding (Fitt et al., 1984) and begin shell formation, and this has similarly been observed in other marine invertebrates such as the sand dollar *S. mirabilis* (Kashenko, 2009) and sea urchin *E. cordatum* (Kashenko, 2007a).

4.3. Tolerance thresholds of cleaved embryos and trochophore larvae

T. gigas larvae showed increased tolerance to hyposaline conditions with age (i.e., larvae > embryos > eggs). Larvae subjected to reduced salinities were able to metamorphose into veligers, whereas eggs and embryos failed to develop to this stage (Fig. 4). However, total mortality was still observed at salinities below 26 ppt regardless of what age they were exposed (Figs. 3, 4 and 5). This suggests that low salinity has a greater effect on the life cycle between embryo and trochophore than the stages between trochophore and veliger larvae. The increase in tolerance with age is similar to the report of Yaroslavtseva and Sergeeva (2009) where trochophore larvae of Pacific mussel *C. grayanus* exposed for nine days to 18 and 20 ppt showed retarded growth while growth of early veliger to veliger was not affected. In addition, a previous study has shown that four day-old *T. gigas* veligers subjected for three days to salinities of 20 and 25 ppt had survival rates of only 1.1% and 2.2%, respectively (Blidberg, 2004). The same study also revealed that, although the proportion of developed larvae at reduced salinities was not statistically different, the number of larvae remaining in free swimming stage was higher compared to larvae exposed to 32 ppt (Blidberg, 2004). Moreover, Maboloc et al. (2014, 2015) reported that two-year old juvenile *T. gigas* cannot tolerate two-week exposure to 18 ppt. Taken together, these findings suggest that the physiological limits of *T. gigas* differ across developmental stages, where older larvae and juveniles tend to have a greater adaptive capacity than cleaved embryos or gametes.

The increase in developmental capacity and resistance with age could be due to more advanced structures and functions, such as shell

formation (Zhang et al., 2012; Dyachuk, 2018), developed cilia (Armstrong et al., 2012), and osmoregulatory structures (e.g., nephridia; Ushakova and Sarantchova, 2004). The reduction in sensitivity to temperature stress with age has similarly been demonstrated in the bivalves *Crassostrea virginica*, *Mulinia lateralis*, *Argopecten irradians*, *Mercenaria mercenaria*, and *Spisula solidissima* (Wright et al., 1983), as well as in the sand dollar *Scaphechinus mirabilis* and the sea star *Asterina pectinefera* (Kashenko, 2007b, 2009). Mak and Chan (2018) proposed that between-stage differences should be further investigated in order to identify the "weakest link" that limits recruitment and survival of the species.

4.4. Larval settlement

Larval settlement is defined when no swimming larvae is observed in the water column (Southgate et al., 2016). *Tridacna gigas* pediveliger larvae that were allowed to settle at a range of salinities showed no significant differences in settlement rate and shell size after four days of exposure (Figs. 6 and 7). Similarly, survival and shell size of 24 h old D-stage larvae of the oyster *Crassostrea iredalei* did not differ significantly after 11 days of culture at salinities of 15 to 30 ppt, which was hypothesized to be due to the presence of their protective shells (Fang et al., 2016). However, Gireesh and Gopinathan (2004) reported that settlement, growth, and survival of *Paphia malabarica* was significantly lower at salinity below 25 ppt. Bivalves at the veliger larvae stage can already close their valves to avoid exposure of their body to a stressful environment and this behavior helps them to sink faster to the bottom where salinity is higher and relatively stable (LaBarbera, 1974; Qiu et al., 2002). However, clams can only close their valves for a short period of time (i.e., juvenile *T. gigas* open after 3 h and 6 h at salinities 25 and 18 ppt respectively, Maboloc et al., 2014) as prolonged closure will result to hypoxia (Kim et al., 2001). Although settled larvae appeared to behave as normal even after four days of exposure to reduced salinities, e.g., crawling on the substrate (Neo et al., 2009), this does not guarantee that they will continue to develop normally and survive, especially if exposure to salinity stress is prolonged. Even if conditions improve, prior exposure of larvae to salinity stress might have a carry-over effect on further development and survival.

4.5. Conclusions

Salinity change is one of the consequences of climate change but has not been given much attention (Chui et al., 2016). It is projected that rainfall events would be more frequent and intensified by monsoons along with the rising sea surface temperature (SST) (Webster et al., 2005; Christensen et al., 2007, 2013; Knutson et al., 2010). This confluence of events might result in significant reduction in seawater salinity that would eventually affect shallow water marine organisms such as giant clams (Blidberg, 2004; Southgate et al., 2016). This study has shown that exposure to reduced salinities has a detrimental effect on the fertilization of eggs and subsequent larval development and survival of the giant clam, *Tridacna gigas*. However, pediveliger settlement rates and shell length were not affected by the exposure to reduced salinity. These results highlight the negative impact of reduced salinity on the progression of embryonic development and metamorphosis in *T. gigas*.

Given that the Philippines is experiencing increasing intensity and frequency of precipitation in some parts of the country (Cinco et al., 2014; Villafuerte et al., 2014), reduced seawater salinity could become a more common phenomenon. This would result in greater chances of exposure of the planktonic larval stages of *T. gigas* to hyposaline conditions, such as in the upper layer of the water column or when pelagic larvae are brought by currents to waters with reduced salinities, which could impede their survival on the reef. Further studies on multiple and interactive stressors that might have synergistic effects on the normal functioning of giant clams would be of interest to better understand their ecological requirements and adaptability to a changing environment.

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